Transcriptional regulation of cell proliferation competence-associated Arabidopsis genes, CDKA;1, RID1 and SRD2, by phytohormones in tissue culture

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Abstract During organ regeneration, differentiated cells acquire cell proliferation competence before the re-start of cell division. In Arabidopsis thaliana (Arabidopsis), CDKA;1, a cyclin-dependent kinase, RID1, a DEAH-box RNA helicase, and SRD2, a small nuclear RNA transcription factor, are implicated in the regulation of cell proliferation competence. Here, we report phytohormonal transcriptional regulation of these cell proliferation competence-associated genes during callus initiation. We can induce the callus initiation from Arabidopsis hypocotyl explants by the culture on the auxin-containing medium. By RT-quantitative PCR analysis, we observed higher mRNA accumulation of CDKA;1, RID1, and SRD2 in culture on the auxin-containing medium than in culture on the auxin-free medium. Promoter-reporter analysis showed that the CDKA;1, RID1, and SRD2 expression was induced in the stele regions containing pericycle cells, where cell division would be resumed to make callus, by the culture in the medium containing auxin and/or cytokinin. However, the expression levels of these genes in cortical and epidermal cells, which would not originate callus cells, were variable by genes and phytohormonal conditions. We also found that the rid1-1 mutation greatly decreased the expression levels of CDKA;1 and SRD2 during callus initiation specifically at 28°C (restrictive temperature), while the srd2-1 mutation did not obviously decrease the expression levels of CDKA;1 and RID1 regardless of temperature conditions but rather even increased them at 22°C (permissive temperature). Together, our results implicated the phytohormonal and differential regulation of cell proliferation competence-associated genes in the multistep regulation of cell proliferation competence.

Key words: auxin, CDKA;1, cytokinin, RID1, SRD2.

Plants can alter their body plans in response to environmental changes. This developmental plasticity can be tightly linked to the flexible control of cell division in plants; for example, the activation and inactivation of cell division in lateral meristems generate variable patterns of branching and numbers of lateral axes. Additionally, differentiated plant cells can re-enter the cell cycle through dedifferentiation processes, giving rise to adventitious organs. These features of plant development exhibit a striking contrast to animal development, in which cell proliferation occurs according to the pre-fixed body plan and most differentiated cells never revert to the proliferative state (Kareem et al. 2016; Sugimoto et al. 2011).

Molecular biological studies have established that basic machinery of cell proliferation is highly conserved among eukaryotes. The progression of the cell cycle is principally governed by the complexes of cyclin-dependent kinase (CDK)/cyclin in fungi, animals, and plants (Inzé and De Veylder 2006). When quiescent plant cells dedifferentiate to resume cell division, A-type CDK (CDKA) plays a critical role together with D-type cyclins (CYCD) at the point of their entry into the cell cycle. An Arabidopsis thaliana (Arabidopsis) gene encoding CDKA, CDKA;1, was shown to be expressed not only in dividing cells but also in non-dividing cells of root tissues, such as the pericycle and parenchyma of the vascular cylinder (Hemerly et al. 1993; Martinez et al. 1992). To explain these facts, Hemerly et al. (1993) proposed a link between the CDKA;1 expression and

Abbreviations: BM, basal medium; BM+D, basal medium containing 2,4-D; BM+K, basal medium containing kinetin; CDKA;1, CYCLIN-DEPENDENT KINASE A;1; CIM, callus-inducing medium; RID1, ROOT INITIATION DEFECTIVE1; SRD2, SHOOT REDIFFERENTIATION DEFECTIVE2.
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In addition to CDKA;1, two Arabidopsis genes, SRD2 and RID1, have also been shown to associate with the cell proliferation competence (Ohtani and Sugiyama 2005; Ohtani et al. 2013, 2015). The expressions of SRD2 and RID1 were highly up-regulated before the re-start of cell division when callus was induced from hypocotyl explants, and the srd2-1 and rid1-1 mutations, which confer single amino acid substitutions presumably resulting in conditional protein dysfunction, caused temperature-dependent defects in hypocotyl dedifferentiation, as clearly seen in the inhibition of the initiation of cell division (Ohtani and Sugiyama 2005; Ohtani et al. 2013). Molecular genetic analysis revealed that SRD2 encodes an activator of transcription for small nuclear RNA (snRNA), which is involved in pre-mRNA splicing and/or ribosomal RNA maturation (Ohtani 2015, 2017, 2018; Ohtani and Sugiyama 2005), and that RID1 is a DEAH-box RNA helicase functioning in pre-mRNA splicing (Ohtani et al. 2013). Thus, pre-mRNA splicing regulation, the common molecular function between snRNA and RID1, should play important roles in acquisition of cell proliferation competence (Ohtani 2015, 2017, 2018; Ohtani and Wachter 2019).

Previous analysis indicated that the spatial expression patterns of CDKA;1, SRD2, and RID1 are similar in seedlings, i.e. these genes are highly expressed in meristematic regions and stele cells of roots, but not in hypocotyl stele cells (Hemerly et al. 1993; Ohtani et al. 2008, 2010, 2013), suggesting that they are under a common transcriptional control. In this study, to obtain further information on transcriptional regulation of these cell proliferation competence-associated genes, we examined their expression in hypocotyl explants cultured in different phytohormonal conditions.

The transgenic lines of Arabidopsis (ecotype Columbia) carrying the GUS reporter gene directed by the CDKA;1, SRD2, or RID1 promoter (Huang et al. 2003; Ohtani and Sugiyama 2005; Ohtani et al. 2013), and the wild type, srd2-1, and rid1-1 of Arabidopsis (ecotype Landsberg erecta) (Ohtani and Sugiyama 2005; Ohtani et al. 2013) were used as plant materials for gene expression analysis. Seeds were surface-sterilized and sown on the Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 10 g l\(^{-1}\) sucrose, buffered with 0.5 g l\(^{-1}\) 2-(N-morpholino)ethanesulfonic acid at pH 5.7, and solidified with 1.5% (w/v) agar, and plants were grown under a continuous light condition (10–15 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) at 22°C. For callus induction, \(\sim\)0.5 cm long hypocotyl explants were excised from 12-day-old seedlings and cultured on the medium based on Gamborg’s B5 basal medium (BM) containing 2% (w/v) glucose and 0.25% (w/v) gellan gum, and phytohormones [0.1 mg l\(^{-1}\) kinetin in BM + K, 0.5 mg l\(^{-1}\) 2,4-D in BM + D, and 0.5 mg l\(^{-1}\) 2,4-D and 0.1 mg l\(^{-1}\) kinetin in callus-inducing medium (CIM)] buffered to pH 5.7 with 0.05% (w/v) MES (Ohtani and Sugiyama 2005; Ohtani et al. 2008, 2010, 2013, 2015). GUS activity detection was performed according to the methods in Ohtani and Sugiyama (2005) and in Ohtani et al. (2013). For the RT-quantitative PCR (RT-qPCR) analysis, the hypocotyl explants derived from 14-day-old seedlings were collected immediately after excision or after being cultured on BM + K, BM + D, or CIM for 14 h at 22°C, and in the case for CIM culture, additionally at 28°C, where, for the srd2-1 and rid1-1 mutants, 22°C and 28°C...
were set as permissive and restrictive temperatures, respectively. Then samples were subjected to total RNA extraction using Plant RNA Purification Reagent (Invitrogen), followed by the purification by RNeasy Mini Kit (QIAGEN) and RNA Clean & Concentration™ (ZYMOL RESEARCH). RT-qPCR analysis was performed by the methods described in Ohtani and Sugiyama (2005) and in Ohtani et al. (2015). The primer sequences and PCR conditions were shown in Supplementary Table S1. All experiments were repeated more than 3 times.

When the hypocotyl explants were cultured on the medium without phytohormone (BM), with kinetin as cytokinin (BM+K), 2,4-D as auxin (BM+D), or both of kinetin and 2,4-D (CIM), callus induction was observed in BM+D and CIM (Figure 1A), indicating that callus initiation requires 2,4-D in the medium (Ohtani and Sugiyama 2005). The RT-qPCR analysis of hypocotyl explants cultured for 1 day, when the cell division does not yet resume even in BM+D or CIM, indicated that the mRNA levels of CDKA;1, RID1, and SRD2 were higher in the BM+D and CIM cultures than in the BM+K culture (Figure 1B). These results suggest that the regulation for the cell proliferation competence-associated genes by 2,4-D would be important prior to the resumption of cell proliferation during callus formation.

Next, we examined the changes in the promoter activity of CDKA;1, RID1, and SRD2 genes in response to phytohormonal conditions in the medium with their GUS reporter lines (Figure 2 and Supplementary Figure S1). For all three GUS reporter lines, the GUS activity was not detectable when cultured on BM (Figure 2). On the other hands, the culture on the medium containing 2,4-D as auxin and/or kinetin as cytokinin induced the GUS activity in all of the reporters, although the GUS activity induction by the BM+K culture was only evident after 2 days of culture (Figure 2). We could recognize basically common expression patterns for these genes; relatively low level of GUS signals was detected in the stele region of hypocotyls cultured on BM+K (containing only kinetin), while the GUS signals in the stele region became more evident in the hypocotyls cultured on BM+D and CIM, which both contained 2,4-D (Figure 2). This result also showed a good correlation between the expression level of these cell proliferation competence-associated genes and the resumption of cell division, as callus initiation from the stele is observed only in the medium containing 2,4-D (i.e. BM+D and CIM) (Ohtani and Sugiyama 2005; Figure 1).

Moreover, we noticed that the application of kinetin in addition to 2,4-D differently affected the expression level and patterns among genes as one can see from the comparison between BM+D and CIM in Figure 1B and Figure 2. The expression level of CDKA;1 in the whole explants was lower in the BM+D culture than that in the CIM culture (Figure 1A), while the CDKA;1 expression in the epidermal and cortical regions was higher in the BM+D culture than that in the CIM culture (Figure 2). However, the RID1 expression was simply increased in the CIM culture as compared with the BM+D culture throughout the explants (Figures 1A and 2). The spatial expression pattern of the SRD2 promoter-directed GUS reporter was almost similar between the BM+D and CIM cultures with the GUS signal in the stele region being somewhat higher in CIM after 1 day of culture (Figure 2). These results indicated that combinational effect of auxin and cytokinin would be different among cell proliferation competence-associated genes. Notably, in spite of the variation of expression patterns in epidermal and cortex regions, the upregulation of CDKA;1, SRD2, and RID1 was commonly observed in the stele region in all culture media tested (Figure 2). These regions contain cells that can form callus, and therefore it seems possible to say that the transcriptional regulation of CDKA;1, SRD2, and RID1 may share common regulatory pathway mediated
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by phytohormonal signals, at least partly, for callus initiation.

The expression level of CDKA;1 at 0 day of culture appeared to be quite different between the RT-qPCR analysis and the GUS reporter analysis; the RT-qPCR data showed a relatively high level of CDKA;1 expression at 0 day of culture comparable to that of the 1-d-cultured explants (Figure 1B), although the CDKA;1 promoter-GUS signals were not detected at 0 day of culture (Figure 2). As CDKA;1 expression is known to be very rapidly and strongly induced by wounding stress (Hemerly et al. 1993), the RT-qPCR analysis might detect the immediate increase of CDKA;1 mRNA level in response to wounding stress associated with hypocotyl excision. In contrast, in the case of GUS reporter analysis, the GUS activity signal is detectable only after the increased GUS mRNA is translated into the GUS protein. This difference in the time required for the detection after the mRNA increase might account for the method-dependent apparent variation in the level of CDKA;1 expression at 0 day of culture.

We also examined effects of srd2-1 and rid1-1 mutations on the mRNA accumulations of CYCB1;2, CDKA;1, SRD2, and RID1 during callus initiation (Figure 3). The CYCB1;2 expression level was higher in srd2-1 and rid1-1 hypocotyls than in the wild-type hypocotyl before CIM culture (Figure 3). After the 24-h CIM culture, the CYCB1;2 expression was upregulated in the wild type regardless of the temperature, but not in srd2-1 nor rid1-1 at 28°C, as reflecting the inhibition of callus initiation at this temperature in the mutants. In accordance with poor cell proliferation in rid1-1 callus even at 22°C (Ohtani et al. 2013), the CYCB1;2 expression was not upregulated in the rid1-1 hypocotyls cultured at 22°C for 24 h (Figure 3). The CDKA;1 and SRD2 expression levels after 1 day of the CIM culture were greatly decreased in rid1-1 specifically at 28°C, whereas the expression levels of CDKA;1 and RID1 were not obviously decreased, but rather even increased at 22°C, by the srd2-1 mutation (Figure 3). In contrast, the expression patterns of RID1 were basically similar among the wild-type, srd2-1, and rid1-1 hypocotyls (Figure 3). These results would suggest that cell proliferation competence-associated genes would be at least partially regulated by each other.

It can be logically supposed that there are at least two distinct states in non-dividing plant cells for each cell proliferation competence-associated gene: the state with or without the sufficient level of gene expression for cell

Figure 3. Expression of CYCB1;2, CDKA;1, RID1 and SRD2 genes during CIM culture of hypocotyl explants in the wild type, srd2-1 and rid1-1. Hypocotyl explants of the wild type (WT), srd2-1, and rid1-1 were excised from 12-d-old seedlings grown at 22°C and immediately collected (0 day of culture) or cultured on CIM at 22°C or 28°C for 1 day. Total RNA was isolated from cultured explants and examined for CYCB1;2, CDKA;1, RID1, and SRD2 mRNA contents by RT-qPCR. The expression level was normalized with UBIQUITIN10, and shown as the relative value to the samples at 0 day of culture. Results were means ± SD (n = 3). Different characters indicate statistical difference (Tukey–Kramer test, p < 0.05).
division resumption. CDKA:1 is one of central regulators of cell cycle (Inzé and De Veylder 2006), and the single mutation of SRD2 or RID1 severely inhibits callus formation at high temperature conditions (Ohtani and Sugiyama 2005; Ohtani et al. 2013, 2015). We found that the CDKA:1 and RID1 genes were strongly upregulated in the epidermal and cortical regions in the BM+D and CIM cultures (Figure 2), although cells of these regions cannot be founder cells of callus in our culture system (Figure 1). All of CDKA:1, SRD2, and RID1 were strongly co-expressed only in the stele, where callus is originated. Taken together, it is likely that fulfillment of a certain level of gene expression for each of CDKA:1, SRD2, and RID1 is required for the activation of cell division. In other words, before the resumption of cell division, non-dividing plant cells would go through differential levels of cell proliferation competence, which can be recognized by differential expression levels of multiple cell proliferation competence-associated genes, including CDKA:1, SRD2, and RID1. Such multistep regulation of cell proliferation competence possibly links to flexible and adaptive regulation of cell division resumption of plant organs, in response to developmental and environmental cues mediated by phytohormonal signaling; indeed, critical roles for auxin, cytokinin, and ethylene have been reported for the resumption of cell division after wounding of tissues (Asahina et al. 2011; Iwase et al. 2011).

Our data also showed that the additional application of cytokinin to auxin differently affected the expression levels and patterns of CDKA:1, SRD2, and RID1 (Figures 1B and 2). This finding suggests that the phytohormone-dependent regulatory pathways should be at least partially different between these cell proliferation competence-associated gene expressions. Future detailed analysis on transcriptional regulation of the cell proliferation competence-associated genes, especially their upstream transcription factors, will provide novel clues to understand the molecular mechanisms of flexible organ regeneration in plants.

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